

TITLE OF THE INVENTION
ASSAY METHODS FOR STATE-DEPENDENT CALCIUM CHANNEL
AGONISTS/ANTAGONISTS

5 CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/418,017, filed October 10, 2003, the contents of which are incorporated herein by reference in their entirety.

10 FIELD OF THE INVENTION

The present invention is directed to methods and cells for studying the effect of candidate compounds on the activity of calcium channels. The methods utilize cells that express a calcium channel of interest and which express a potassium channel. The engineered cells allow for fine control of the membrane potential of the cells, which, in turn, provide a high
15 resolution assay for studying the effects of targeted compounds at various states of the calcium channel.

BACKGROUND OF THE INVENTION

Certain molecular events in eukaryotic cells depend on the existence or magnitude
20 of an electric potential gradient across the plasma (i.e., outer) membrane of the cells. Among the more important of such events is the movement of ions across the plasma membrane through voltage-gated ion channels. Voltage-gated ion channels form transmembrane pores that open in response to changes in cell membrane potential and allow ions to pass through the membrane. Voltage-gated ion channels have many physiological roles. They have been shown to be
25 involved in maintaining cell membrane potentials and controlling the repolarization of action potentials in many types of cells (Bennett et al., 1993, Cardiovascular Drugs & Therapy 7:195-202; Johnson et al., 1999, J. Gen. Physiol. 113:565-580; Bennett & Shin, "Biophysics of voltage-gated sodium channels," in Cardiac Electrophysiology: From Cell to Bedside, 3rd edition, D. Zipes & J. Jalife, eds., 2000, W.B. Saunders Co., pp.67-86; Bennett & Johnson, "Molecular
30 physiology of cardiac ion channels," Chapter 2 in Basic Cardiac Electrophysiology and Pharmacology, 1st edition, A. Zasa & M. Rosen, eds., 2000, Harwood Academic Press, pp. 29-57). Moreover, mutations in sodium, calcium, or potassium voltage-gated ion channel genes leading to defective channel proteins have been implicated in a variety of disorders including the congenital long QT syndromes, ataxia, migraine, muscle paralysis, deafness, seizures, and
35 cardiac conduction diseases, to name a few (Bennett et al., 1995, Nature 376:683-685; Roden et

al., 1995, *J. Cardiovasc. Electrophysiol.* 6:1023-1031; Kors et al., 1999, *Curr. Opin. Neurol.* 12:249-254; Lehmann et al., 1999, *Physiol. Rev.* 79:1317-1372; Holbauer & Heufelder, 1997, *Eur. J. Endocrinol.* 136:588-589; Naccarelli & Antzelevitch, 2000, *Am. J. Med.* 110:573-581).

Several types of voltage-gated ion channels exist. Voltage-gated potassium channels establish the resting membrane potential and modulate the frequency and duration of action potentials in neurons, muscle cells, and secretory cells. Following depolarization of the membrane potential, voltage-gated potassium channels open, allowing potassium efflux and thus membrane repolarization. This behavior has made voltage-gated potassium channels important targets for drug discovery in connection with a variety of diseases. Dysfunctional voltage-gated potassium channels have been implicated in a number of diseases and disorders. Wang et al., 1998, *Science* 282:1890-1893 have shown that the voltage-gated potassium channels KCNQ2 and KCNQ3 form a heteromeric potassium ion channel known as the "M-channel." Mutations in KCNQ2 and KCNQ3 in the M-channel are responsible for causing epilepsy (Biervert et al., 1998, *Science* 279:403-406; Singh et al., 1998, *Nature Genet.* 18:25-29; Schroeder et al., *Nature* 1998, 396:687-690).

Voltage-gated sodium channels are transmembrane proteins that are essential for the generation of action potentials in excitable cells (Catterall, 1993, *Trends Neurosci.* 16:500-506). In mammals, voltage-gated sodium channels consist of a macromolecular assembly of α and β subunits with the α subunit being the pore-forming component. α subunits are encoded by a large family of related genes, with some α subunits being present in the central nervous system (Noda et al., 1986, *Nature* 322:826-828; Auld et al., 1988, *Neuron* 1:449-461; Kayano et al., 1988, *FEBS Lett.* 228:187-194) and others in muscle (Rogart et al., 1989, *Proc. Natl. Acad. Sci. USA* 86:8170-8174; Trimmer et al., 1989, *Neuron* 3:33-49).

Voltage-gated calcium channels are transmembrane proteins that in the open configuration allow the passive flux of Ca^{2+} ions across the plasma membrane, down the electrochemical gradient. They mediate various cell functions, including excitation-contraction coupling, signal transduction, and neurotransmitter release. Three major classes of calcium channel antagonists including the dihydropyridines, benzothiazepines and phenylalkylamines have been widely used clinically in the treatment of cardiovascular diseases. These drugs antagonize the L-type calcium channels found throughout the body, including the cardiovascular system. Calcium channels are allosteric proteins that undergo changes in conformational state. The distinct conformational states of these proteins have different affinities for ligands, including these antagonists. Membrane potential is an allosteric effector of these conformational changes in ion channel proteins. The potency of inhibition by these calcium channel antagonists is dependent on the state of the calcium channel. Previously studies on state-dependent

interactions of these antagonists were identified through voltage clamp (1), radioligand binding (2) and cell based, e.g. smooth muscle contraction (3) studies. While each of these methods yields valuable information each has its drawbacks in terms of information content or throughput, respectively.

5 Calcium channels are membrane-spanning, multi-subunit proteins that allow controlled entry of Ca^{+2} ions into cells from the extracellular fluid. Cells throughout the animal kingdom, and at least some bacterial, fungal and plant cells, possess one or more types of calcium channel.

10 The most common type of calcium channel is voltage dependent. Most "excitable" cells in animals, such as neurons of the central nervous system (CNS), peripheral nerve cells and muscle cells, including those of skeletal muscles, cardiac muscles, and venous and arterial smooth muscles, have voltage-dependent calcium channels. "Opening" of a voltage-dependent channel to allow an influx of Ca^{+2} ions into the cells requires a depolarization to a certain level of the potential difference between the inside of the cell bearing the channel and the
15 extracellular environment bathing the cell. The rate of influx of Ca^{+2} into the cell depends on this potential difference.

Multiple types of calcium channels have been identified in mammalian cells from various tissues, including skeletal muscle, cardiac muscle, lung, smooth muscle and brain, [see, e.g., Bean, B. P. (1989) *Ann. Rev. Physiol.* 51:367-384 and Hess, P. (1990) *Ann. Rev. Neurosci.*
20 56:337]. The different types of calcium channels have been broadly categorized into five classes, L-, T-, N-, P/Q and R-type, distinguished by current kinetics, holding potential sensitivity and sensitivity to calcium channel agonists and antagonists.

Current methods of drug discovery often involve assessing the biological activity (i.e., screening) of tens or hundreds of thousands of compounds in order to identify a small
25 number of those compounds having a desired activity. In many high throughput screening programs, it is desirable to test as many as 50,000 to 100,000 compounds per day. Unfortunately, current methods of assaying the activity of voltage-gated ion channels are ill suited to the needs of a high throughput screening program. Current methods often rely on electrophysiological techniques. Standard electrophysiological techniques involve "patching" or
30 sealing against the cell membrane with a glass pipette followed by suction on the glass pipette, leading to rupture of the membrane patch (Hamill et al., 1981, *Pflugers Arch.* 391:85-100). This has limitations and disadvantages. Accessing the cell interior may alter the cell's response properties. The high precision optical apparatuses necessary for micromanipulating the cells and the pipettes make simultaneous recording from more than a few cells at a time impossible.

Given these difficulties, the throughput that can be achieved with electrophysiological techniques falls far short of that necessary for high throughput screening.

Various techniques have been developed as alternatives to standard methods of electrophysiology. For example, radioactive flux assays have been used in which cells are exposed with a radioactive tracer (e.g., $^{86}\text{Rb}^+$, $^{22}\text{Na}^+$, [^{14}C]-guanidinium and ^{45}Ca) and the flux of the radio-labeled ion is monitored. Cells loaded with the tracer are exposed to compounds and those compounds that either enhance or diminish the efflux of the tracer are identified as possible activators or inhibitors of ion channels in the cells' membranes.

Assays that measure the change in a cell's membrane potential due to the change in activity of an ion channel have been developed. Such assays often employ voltage sensitive dyes that redistribute between the extracellular environment and the cell's interior based upon a change in membrane potential and that have a different fluorescence spectrum depending on whether they are inside or outside the cell. A related assay method uses a pair of fluorescent dyes capable of fluorescence resonance energy transfer to sense changes in membrane potential. For a description of this technique, see González & Tsien, 1997, *Chemistry & Biology* 4:269-277. See also González & Tsien, 1995, *Biophys. J.* 69:1272-1280 and U.S. Patent No. 5,661,035. Other methods employ ion selective indicators such as calcium dependent fluorescent dyes to monitor changes in Ca^{2+} influx during opening and closing of calcium channels.

Ideally, methods of screening against voltage-gated ion channels require that the transmembrane potential of the cells being assayed be controlled and/or that the ion channels studied be cycled between open and closed states. This has been done in various ways. In standard electrophysiological techniques, the experimental set-up allows for direct manipulation of membrane potential by the voltage clamp method (Hodgkin & Huxley, 1952, *J. Physiol. (Lond.)* 153:449-544), e.g., changing the applied voltage. In other methods, changing the extracellular K^+ concentration from a low value (e.g., 5 mM) to a higher value (e.g., 70-80 mM) results in a change in the electrochemical potential for K^+ due to the change in the relative proportion of intracellular and extracellular potassium. This results in a change in the transmembrane electrical potential towards a more depolarized state. This depolarization can activate many voltage-gated ion channels, e.g., voltage-gated calcium, sodium, or potassium channels. Alternatively, Na^+ channels can be induced into an open conformation by the use of toxins such as veratridine or scorpion venom (Strichartz et al., 1987, *Ann. Rev. Neurosci.* 10:237-267; Narahashi & Harman, 1992, *Meth. Enzymol.* 207:620-643). While sometimes effective, such experimental manipulations may alter the channel pharmacology, can be awkward to perform, and can lead to artifactual disturbances in the system being studied.

Electrical field stimulation (EFS) has been used to activate ion channels. In this approach, membrane potential is altered but not controlled. The uncertainty and lack of control of membrane potential make EFS a less than optimal method for the study of ion channels.

HEK293 cells have been grown on a silicon chip made up of an array of field-effect transistors. Some of the cells were positioned over the gate region of the transistors, thus having portions of their plasma membranes overlying the source and the drain. When a patch pipette in such cells manipulated the intracellular voltage, Maxi-K potassium channels in the cells' plasma membranes were opened. This led to current flow in the region between the cells' membrane and the transistor. This current flow modulated the source-drain current, which could be detected by an appropriate device. The chip plus cells was said to have potential as a sensor and as a prototype for neuroprosthetic devices. See Straub et al., 2001, Nature Biotechnol. 19:121-124; Neher, 2001, Nature Biotechnol. 19:114.

SUMMARY OF THE INVENTION

The present invention is directed to methods of identifying activators and inhibitors of voltage-gated ion channels, and specifically calcium ion channels. The methods employ cells transformed to express a voltage-gated calcium ion channel of interest and an inward rectifier potassium channel. The addition of the potassium channel allows for the fine control of the membrane potential of the cells. Manipulation of the extracellular potassium concentration controls the membrane potential which in turn affects the open/close state transitions of the voltage-gated ion channels. This allows for more convenient, more precise manipulation of these transitions, and, coupled with efficient methods of detecting ion flux, results in methods that are especially suitable for high throughput screening in order to identify substances that are channel state dependent modulators of voltage-gated ion channels.

According to a specific embodiment, the present invention describes the state-dependent interactions of the calcium channel antagonists directly in a functional cell-based FLIPR (Fluorometric Imaging Plate Reader) assay, which measures calcium influx through a voltage-dependent calcium channel (VDCC). The cell line used in this embodiment has a stably transfected L-type calcium channel, the $\alpha 1 C$ subunit. It also was transfected with the Kir 2.3 inward rectifier K channel, which allows for control of cell membrane potential through alteration of extracellular $[K^+]_o$. Preincubation of the cells for 10 min in 30 mM $[K^+]_o$ partially depolarizes the cells. The inhibitory effect of calcium channel antagonists on calcium influx in response to a high $[K^+]_o$ depolarization (final $[K^+]_o$ 85.8 mM) was shifted to the left compared with that observed for cells in normal, physiological $[K^+]_o$ (5.8 mM). The ratio of IC_{50} values between the potencies for the antagonists tested in the normally polarized and depolarized cells

was 4 to 20-fold. The results suggest that the interaction of these calcium channel antagonists with the channel expressing cells is dependent upon the state of the channel, which is modulated by changes in membrane potential. The state dependent assay demonstrated in these studies is useful for evaluating state dependent inhibitory potency of a large number of samples and can be used to identify state-dependent calcium channel antagonists.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows immunostaining of $\alpha 1C$ subunit in the wild type HEK 293 cells and cells stably transfected with the L-type $\alpha 1C$ channel (C1-6-37-3).

Figure 2 shows immunostaining of Kir2.3 subunit in the wild type HEK293 cells and the cells stably transfected with the L-type $\alpha 1C$ channel (C1-6-37-3).

Figure 3 is a graph showing the relationship between extracellular potassium ($[K]_o$) and cell membrane potential. Three situations are shown. One is the prediction of the Nernst equation for a perfectly K-selective membrane. The other curves show the effects of partial permeability by other ions, Na^+ and/or Cl^- . Membrane potential can be set in a non-voltage clamped cell by adjusting external potassium.

A cell line expressing an inward rectifier K channel (Kir2.3) to set the resting membrane potential will permit control of membrane resting potential by extracellular potassium.

Figure 4 is a graph demonstrating the dose-response relationship for K^+ -stimulated calcium influx in wild type HEK 293 cells and cells stably transfected with the L-type $\alpha 1C$ channel (C1-6-37-3).

Figure 5 is a graph demonstrating a comparison of nimodipine and mibefradil inhibition curves in K^+ -stimulated calcium influx in C1-6-37-3 cells under resting condition (5.8 mM K, -65 mV).

Figure 6 is a graph representing the nimodipine inhibition curve stimulated by K^+ (final 85.8 mM) either in 30 mM K^+ (depolarized condition, -28 mV) or 5.8 mM K^+ (resting condition, -65 mV).

Figure 7 is summary table of IC_{50} (nM) values for calcium channel antagonists in 30 mM K^+ (depolarized condition, -28 mV) and 5.8 mM K^+ (resting condition, -65 mV).

DETAILED DESCRIPTION OF THE INVENTION

Without intending to bound by any theory, voltage gated calcium channels open as a function of membrane potential such that the probability of opening increases with membrane depolarization. Voltage gated calcium channels inactivate (close / desensitize) as a

function of membrane potential such that the probability of inactivation increases with membrane depolarization. These steady state voltage dependent processes overlap. Changes in membrane potential populate different conformational states of these channels (closed, open or inactivated). Drug binding to voltage gated calcium channels is often channel state dependent
5 such that more or less binding occurs depending upon the state occupied. Control of membrane potential, permits channels to be manipulated into various states. This membrane potential control is typically achieved by voltage clamp electrophysiology methods, but this method is not at present amenable to high throughput drug screening.

Specifically exemplified herein is an assay to determine state-dependent drug-
10 calcium channel interactions using a cell line that co-expresses a potassium channel (Kir2.3) that determines the resting membrane potential of the cells as a function of the external potassium ion concentration ([K]_o) and a voltage gated calcium channel. Co-expressed in these cells is the L-type voltage gated calcium channel complex (alpha1C, alpha2-delta, beta2a). Potassium is used in a two step manner in this assay. First it is used to set the resting membrane potential (V_m)
15 during antagonist incubation. Two conditions were selected for illustration purposes, polarized and depolarized resting conditions. In the polarized resting condition, cells are incubated in 5.8 mM [K]_o to set the membrane potential to -65 mV (V_m as a function of [K]_o). Drugs exposed to these cells will bind to calcium channels primarily in the closed, rested, low affinity state. In order to reveal higher affinity states of the calcium channels, the cells are incubated in 30 mM
20 [K]_o, in order to chronically and partially depolarize them to -28 mV during drug exposure. This change in the membrane potential, shifts the calcium channels into the higher affinity inactivated states and antagonist binding is enhanced. Upon establishing these two different conditions for drug exposure, channels are then forced to open by further depolarization to near 0 mV by exposure to 85.8 mM [K]_o. Opening of these channels normally under control, non-
25 antagonist exposed conditions, allows calcium influx into the cells. This calcium influx is detected using a calcium sensitive dye (eg Fluo-3, Fluo-4, Fura2, etc.). If the calcium influx is diminished by exposure to antagonists, this will be detected when compared to the control condition. In some cases, antagonists will bind with greater affinity to the channels in the depolarized (30 mM [K]_o) condition. In these cases, the same drug will appear more potent
30 under these depolarized assay conditions. This approach creates a novel high throughput calcium channels assay system that is capable of detecting and measuring calcium channel state dependent drug interactions as have been described using low throughput voltage clamp measures on single cells.

This foregoing approach and the referenced cells have been tested using
35 conventional voltage- and current- clamp methods, and the membrane potential changes as a

function [K]_o and the state dependent calcium current and drug affinities have been confirmed experimentally. The foregoing approach can be modified as taught herein to study state-dependencies of agonists/antagonists for many different types of ion channels.

5 In one embodiment, the present invention involves providing a substrate upon which living eukaryotic cells, preferably mammalian cells, are present where the cells express voltage-gated calcium ion channels in their plasma membranes. Upon application of varying concentrations of extracellular calcium, voltage-gated ion channels either open or close, thereby modulating the flow of at least one type of ion through the plasma membranes of the cells. This modulation of ion flow, or a change in membrane potential that results from the modulation of
10 ion flow, is detected, either directly or indirectly, preferably by the use of fluorescent indicator compounds in the cells. Collections of substances, *e.g.*, combinatorial libraries of small organic molecules, natural products, phage display peptide libraries, etc., are brought into contact with the voltage-gated ion channels in the plasma membranes of the cells and those substances that are able to affect the modulation of ion flow are identified. In this way, the present invention
15 provides methods of screening for activators and inhibitors of voltage-gated ion channels, particularly calcium channels. Such activators and inhibitors are expected to be useful as pharmaceuticals or as lead compounds from which pharmaceuticals can be developed by the usual processes of drug development, *e.g.*, medicinal chemistry.

20 Accordingly, the present invention provides a method for identifying modulators of the activity of a voltage-gated calcium ion channel comprising:

- (a) providing cells expressing the voltage-gated calcium ion channel and expressing an inward rectifying potassium channel;
- (b) dividing the cells into group 1 and group 2;
- (c) changing extracellular potassium concentration of the group 2;
- 25 (c) exposing the cells of groups 1 and 2 to a substance of interest;
- (d) depolarizing the cells of groups 1 and 2 while monitoring ion flux through the voltage-gated calcium ion channel;
- (c) comparing the ion flow through the voltage-gated calcium ion channel in groups 1 and 2;

30 where a difference in the ion flow through the voltage-gated calcium ion channel in groups 1 and 2 indicates that the substance is a modulator of the voltage-gated channels, and where the potency of the modulator is affected by the state of the voltage-gated calcium ion channel.

35 For the sake of simplicity, the above methods are described in terms of "a" voltage-gated ion channel although those skilled in the art will understand that in actual practice

the cells will express a plurality of the voltage-gated ion channels for which modulators are sought. Generally, each cell will express at least 10^2 , 10^3 , 10^4 , 10^5 , 10^6 or more molecules of the voltage-gated ion channel. Also, ion flow will be monitored through the plurality of the voltage-gated ion channels rather than through a single voltage-gated ion channel. Similarly, the methods will generally be practiced by employing a plurality of cells, even though the methods are described above in terms of "a" cell.

Generally, the methods of the present invention will be carried out on a substrate that is a modified version of a standard multiwell tissue culture plate or microtiter plate.

The skilled person will recognize that it is generally beneficial to run controls together with the methods described herein. For example, it will usually be helpful to have a control in which the substances are tested in the methods against cells that preferably are essentially identical to the cells that are used in the methods except that these cells would not express the voltage-gated ion channels of interest. In this way it can be determined that substances which are identified by the methods are really exerting their effects through the voltage-gated ion channels of interest rather than through some unexpected non-specific mechanism. One possibility for such control cells would be to use non-recombinant parent cells where the cells of the actual experiment express the voltage-gated ion channels of interest due to the recombinant expression of those voltage-gated ion channels of interest.

Other types of controls would involve taking substances that are identified by the methods of the present invention as activators or inhibitors of voltage-gated ion channels of interest and testing those substances in the methods of the prior art in order to confirm that those substances are also activators and inhibitors when tested in those prior art methods.

One skilled in the art would recognize that, where the present invention involves comparing control values for the flow of ions to test values for the flow of ions and determining whether the control values are greater or less than the test values, a non-trivial difference is sought. For example, if in the methods of identifying inhibitors, the control value were found to be 1% greater than the test value, this would not indicate that the substance is an inhibitor. Rather, one skilled in the art would attribute such a small difference to normal experimental variance. What is looked for is a significant difference between control and test values. For the purposes of this invention, a significant difference fulfills the usual requirements for a statistically valid measurement of a biological signal. For example, depending upon the details of the experimental arrangement, a significant difference might be a difference of at least 10%, preferably at least 20%, more preferably at least 50%, and most preferably at least 100%.

One skilled in the art would understand that the cells that give rise to the control values need not be physically the same cells that give rise to the test values, although that is

possible. What is necessary is that the cells that give rise to the control values be substantially the same type of cell as the cells that give rise to the test values. A cell line that has been transfected with and expresses a certain voltage-gated ion channel could be used for both the control and test cells. Large numbers of such cells could be grown and a portion of those cells could be exposed to the substance and thus serve as the cells giving rise to the test value for ion flow while a portion would not be exposed to the substance and would thus serve as the cells giving rise to the control value for ion flow. No individual cell itself would be both control and test cell but the virtual identity of all the cells in the cell line ensures that the methods would nevertheless be reliable.

“Substances” can be any substances that are generally screened in the pharmaceutical industry during the drug development process. For example, substances may be low molecular weight organic compounds (*e.g.*, having a molecular weight of less than about 1,000 daltons); RNA, DNA, antibodies, peptides, or proteins.

The conditions under which cells are exposed to substances in the methods described herein are conditions that are typically used in the art for the study of protein-ligand interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature preferably of about 18°C to about 45°C; incubation times of from several seconds to several hours. Generally, the cells are present in wells in the substrate and the substances are added directly to the wells, optionally after first washing away the media in the wells.

Determining the values of ion flux in the methods of the present invention can be accomplished through the use of fluorescent indicator compounds. One type of fluorescent indicator compound is sensitive to the level of intracellular calcium ions in the cells used in the present invention. This type of fluorescent indicator compound can be used when the methods are directed to those voltage-gated ion channels whose activity results in a change in intracellular calcium levels. Such voltage-gated ion channels include not only voltage-gated calcium channels but also other types of voltage-gated ion channels where the activity of those channels is naturally or can be coupled to changes in intracellular calcium levels. Many types of voltage-gated potassium channels can be so coupled. When using this approach to study a voltage-gated ion channel of interest that is not a voltage-gated calcium channel, it may be desirable to engineer the cells employed so as to recombinantly express voltage-gated calcium channels that are coupled to the voltage-gated ion channel of interest.

Fluorescent indicator compounds suitable for measuring intracellular calcium levels include various calcium indicator dyes (*e.g.*, fura-2, fluo-3, fluo-4, indo-1, Calcium Green; see Velicelebi et al., 1999, *Meth. Enzymol.* 294:20-47).

Calcium indicator dyes are substances which show a change in a fluorescent characteristic upon binding calcium, *e.g.*, greatly increased intensity of fluorescence and/or a change in fluorescent spectra (*i.e.*, a change in emission or excitation maxima). Fluo-3, fura-2, and indo-1 are commonly used calcium indicator dyes that were designed as structural analogs of the highly selective calcium chelators ethylene glycol-bis(β -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) and 1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA). The fluorescence intensity from fluo-3 increases by more than 100-fold upon binding of calcium. While the unbound dye exhibits very little fluorescence, calcium-bound fluo-3 shows strong fluorescence emission at 526 nm. Fura-2 is an example of a dye that exhibits a change in its fluorescence spectrum upon calcium binding. In the unbound state, fura-2 has an excitation maximum of 362 nm. This excitation maximum shifts to 335 nm upon calcium binding, although there is no change in emission maximum. Binding of calcium to fura-2 can be monitored by excitation at the two excitation maxima and determining the ratio of the amount of fluorescence emission following excitation at 362 nm compared to the amount of fluorescence emission following excitation at 335 nm. A smaller ratio (*i.e.*, less emission following excitation at 362 nm) indicates that more fura-2 is bound to calcium, and thus a higher internal calcium concentration in the cell.

The use of calcium indicator dyes entails loading cells with the dye, a process which can be accomplished by exposing cells to the membrane-permeable acetoxymethyl esters of the dyes. Once inside the plasma membrane of the cells, intracellular esterases cleave the esters, exposing negative charges in the free dyes. This prevents the free dyes from crossing the plasma membrane and thus leaves the free dyes trapped in the cells. Measurements of fluorescence from the dyes are then made, the cells are treated in such a way that the internal calcium concentration is changed (*e.g.*, by exposing cells to an activator or inhibitor of a voltage-gated ion channel), and fluorescence measurements are again taken.

Fluorescence from the indicator dyes can be measured with a luminometer or a fluorescence imager. One preferred detection instrument is the Fluorometric Imaging Plate Reader (FLIPR) (Molecular Devices, Sunnyvale, CA). The FLIPR is well suited to high throughput screening using the methods of the present invention as it incorporates integrated liquid handling capable of simultaneously pipetting to 96 or 384 wells of a microtiter plate and rapid kinetic detection using a argon laser coupled to a charge-coupled device imaging camera.

A typical protocol for use of calcium indicator dyes would entail putting cells expressing a voltage-gated ion channel of interest into an appropriate substrate (*e.g.*, clear, flat-bottom, black-wall 96 well plates) and allowing the cells to grow overnight in standard tissue culture conditions (*e.g.*, 5% CO₂, 37°C). The cells are generally plated at a density of about

10,000 to 100,000 cells per well in appropriate growth medium. On the day of the assay, growth medium is removed and dye loading medium is added to the wells.

5 If the calcium indicator dye is fluo-3, *e.g.*, dye loading medium could be prepared by solubilizing 50 µg of fluo-3-AM ester (Molecular Probes F-1242) in 22 µl DMSO to give a 2 mM dye stock. Immediately before loading the cells, 22 µl 20% pluronic acid (Molecular Probes P-3000) is added to the dye. The tube containing the dye is mixed with a vortex mixer. For one 96-well plate, 44 ml of the dye/pluronic acid solution is added to 10.5 ml of Hanks Balanced Salt Solution (Gibco/BRL Cat # 14025-076) with 20 mM HEPES (Gibco/BRL Cat # 1560-080), and 1% fetal bovine serum (Gibco/BRL Cat # 26140-087; not BSA)). The dye and the loading
10 medium are mixed by repeated inversion (final dye concentration about 4 µM).

Growth medium can be removed from the cells by washing (wash medium is Hanks Balanced Salt Solution (Gibco/BRL Cat # 14025-076) with 20 mM HEPES (Gibco/BRL Cat # 1560-080), and 0.1% bovine serum albumin (Sigma Cat # A-9647; not FBS) two times, leaving 100 µl residual medium in the wells after the second wash. Then 100 µl of the dye in the
15 loading medium is added to each well. The cells are then incubated for 60 minutes at 37°C to allow for dye loading.

Following dye loading, the cells in each well are washed for four times, then fluorescent measurements of the cells are taken prior to exposure of the cells to substances that are to be tested. The cells are then exposed to the substances and those substances that cause a
20 change in a fluorescent characteristic of the dye are identified. The measuring instrument can be a fluorescent plate reader such as the FLIPR (Molecular Devices). Substances that cause a change in a fluorescent characteristic in the test cells but not the control cells are possible activators or inhibitors of the voltage-gated ion channel.

The exact details of the procedure outlined above are meant to be illustrative.
25 One skilled in the art would be able to optimize experimental parameters (cell number, dye concentration, dye loading time, temperature of incubations, cell washing conditions, and instrument settings, etc.) by routine experimentation depending on the particular relevant experimental variables (*e.g.*, type of cell used, identity of dye used). Several examples of experimental protocols that can be used are described in Veliçelebi et al., 1999, Meth. Enzymol.
30 294:20-47. Other suitable instrumentation and methods for measuring transmembrane potential changes via optical methods includes microscopes, multiwell plate readers and other instrumentation that is capable of rapid, sensitive ratiometric fluorescence detection. For example, the VIPR (Aurora Biosciences, San Diego, CA) is an integrated liquid handler and kinetic fluorescence reader for 96-well and greater multiwell plates. The VIPR reader integrates
35 an eight channel liquid handler, a multiwell positioning stage and a fiber-optic illumination and

detection system. The system is designed to measure fluorescence from a column of eight wells simultaneously before, during and after the introduction of liquid sample obtained from another microtiter plate or trough. The VIPR reader excites and detects emission signals from the bottom of a multiwell plate by employing eight trifurcated optical bundles (one bundle for each well). One leg of the trifurcated fiber is used as an excitation source, the other two legs of the trifurcated fiber being used to detect fluorescence emission. A ball lens on the end of the fiber increases the efficiency of light excitation and collection. The bifurcated emission fibers allow the reader to detect two emission signals simultaneously and are compatible with rapid signals generated by the FRET-based voltage dyes. Photomultiplier tubes then detect emission fluorescence, enabling sub-second emission ratio detection.

In particular embodiments, the calcium indicator dye is selected from the group consisting of: fluo-3, fura-2, fluo-4, fluo-5, calcium green-1, Oregon green, 488 BAPTA, SNARF-1, and indo-1.

In particular embodiments, the change in fluorescent characteristic is an increase in intensity of a fluorescence emission maximum. In other embodiments, the change in fluorescent characteristic is a shift in the wavelength of an absorption maximum.

In particular embodiments, the cells naturally express the voltage-gated ion channel of interest. In other embodiments, the cells do not naturally express the voltage-gated ion channel of interest but instead have been transfected with expression vectors that encode the voltage-gated ion channel of interest so that the cells recombinantly express the voltage-gated ion channel of interest. Transfection is meant to include any method known in the art for introducing expression vectors into the cells. For example, transfection includes calcium phosphate or calcium chloride mediated transfection, lipofection, infection with a retroviral construct, and electroporation.

An alternative to the use of calcium indicator dyes is the use of the aequorin system. The aequorin system makes use of the protein apoequorin, which binds to the lipophilic chromophore coelenterazine forming a combination of apoequorin and coelenterazine that is known as aequorin. Apoequorin has three calcium binding sites and, upon calcium binding, the apoequorin portion of aequorin changes its conformation. This change in conformation causes coelenterazine to be oxidized into coelenteramide, CO₂, and a photon of blue light (466 nm). This photon can be detected with suitable instrumentation.

Since the gene encoding apoequorin has been cloned (U.S. Patent No. 5,541,309; U.S. Patent No. 5,422,266; U.S. Patent No. 5,744,579; Inouye et al., 1985, Proc. Natl. Acad. Sci. USA 82:3154-3158; Prasher et al., 1985, Biochem. Biophys. Res. Comm. 126:1259-1268), apoequorin can be recombinantly expressed in cells in which it is desired to measure the

intracellular calcium concentration. Alternatively, existing cells that stably express recombinant apoequorin can be used. Such cells derived from HEK293 cells and CHO-K1 cells are described in Button & Brownstein, 1993, *Cell Calcium* 14:663-671. For example, the HEK293/aeq17 cell line can be used as follows.

5 The HEK293/aeq17 cells are grown in Dulbecco's Modified Medium (DMEM, GIBCO-BRL, Gaithersburg, MD, USA) with 10% fetal bovine serum (heat inactivated), 1 mM sodium pyruvate, 500 µg/ml Geneticin, 100 µg/ml streptomycin, 100 units/ml penicillin. Expression vectors encoding the voltage-gated ion channel of interest as well as, optionally, the
10 desired voltage-gated calcium channel subunits (α_1A , α_1B , α_1C , α_1D , α_1E , α_1G , α_1H , α_1I , $\alpha_2\delta$, β_1 , β_2 , β_3 , β_4 , etc.) can be transfected into the HEK293/aeq17 cells by standard methods in order to express the desired voltage-gated ion channel subunits and voltage-gated calcium channel subunits in the HEK293/aeq17 cells. The cells are washed once with DMEM plus 0.1 % fetal bovine serum, and then charged for one hour at 37°C /5% CO₂ in DMEM containing 8 µM coelenterazine cp (Molecular Probes, Eugene, OR, USA) and 30 µM glutathione. The cells are
15 then washed once with Versene (GIBCO-BRL, Gaithersburg, MD, USA), detached using Enzyme-free cell dissociation buffer (GIBCO-BRL, Gaithersburg, MD, USA), diluted into ECB (Ham's F12 nutrient mixture (GIBCO-BRL) with 0.3 mM CaCl₂, 25 mM HEPES, pH7.3, 0.1% fetal bovine serum). The cell suspension is centrifuged at 500 x g for 5 min. The supernatant is removed, and the pellet is resuspended in 10 ml ECB. The cell density is determined by
20 counting with a hemacytometer and adjusted to 500,000 cells/ml in ECB. The substances to be tested are diluted to the desired concentrations in ECB and aliquoted into the assay plates, preferably in triplicate, at 0.1 ml/well. The cell suspension is injected at 0.1 ml/well, read and integrated for a total of 400 readings using a luminometer (Luminoskan Ascent, Labsystems Oy, Helsinki, Finland). Alternatively, the cells may first be placed into the assay plates and then the
25 substances added. Data are analyzed using the software GraphPad Prism Version 3.0 (GraphPad Software, Inc., San Diego, CA, USA).

 It will be understood by those skilled in the art that the procedure outlined above is a general guide in which the various steps and variables can be modified somewhat to take into account the specific details of the particular assay that is desired to be run. For example, one
30 could use semisynthetic coelenterazine (Shimomura, 1989, *Biochem. J.* 261:913-920; Shimomura et al., 1993, *Cell Calcium* 14:373-378); the time of incubation of the cells with coelenterazine can be varied somewhat; somewhat greater or lesser numbers of cells per well can be used; and so forth.

 For reviews on the use of aequorin, see Créton et al., 1999, *Microscopy Research and Technique* 46:390-397; Brini et al., 1995, *J. Biol. Chem.* 270:9896-9903; Knight & Knight,
35

1995, Meth. Cell. Biol. 49:201-216. Also of interest may be U.S. Patent No. 5,714,666 which describes methods of measuring intracellular calcium in mammalian cells by the addition of coelenterazine co-factors to mammalian cells that express apoequorin.

Another way to measure ion flow indirectly is to monitor changes in transcription
5 that result from the activity of voltage-gated ion channels by the use of transcription based assays. Transcription-based assays involve the use of a reporter gene whose transcription is driven by an inducible promoter whose activity is regulated by a particular intracellular event such as, *e.g.*, changes in intracellular calcium levels, that are caused by the activity of a voltage-gated ion channel. Transcription-based assays are reviewed in Rutter et al., 1998, Chemistry &
10 Biology 5:R285-R290. Transcription-based assays of the present invention rely on the expression of reporter genes whose transcription is activated or repressed as a result of intracellular events that are caused by the interaction of a activator or inhibitor with a voltage-gated ion channel.

An extremely sensitive transcription-based assay is disclosed in Zlokarnik et al.,
15 1998, Science 279:84-88 (Zlokarnik) and also in U.S. Patent No. 5,741,657. The assay disclosed in Zlokarnik and U.S. Patent No. 5,741,657 employs a plasmid encoding β -lactamase under the control of an inducible promoter. This plasmid is transfected into cells together with a plasmid encoding a receptor for which it is desired to identify agonists. The inducible promoter on the β -lactamase is chosen so that it responds to at least one intracellular signal that is generated when
20 an agonist binds to the receptor. Thus, following such binding of agonist to receptor, the level of β -lactamase in the transfected cells increases. This increase in β -lactamase is measured by treating the cells with a cell-permeable dye that is a substrate for cleavage by β -lactamase. The dye contains two fluorescent moieties. In the intact dye, the two fluorescent moieties are physically linked, and thus close enough to one another that fluorescence resonance energy
25 transfer (FRET) can take place between them. Following cleavage of the dye into two parts by β -lactamase, the two fluorescent moieties are located on different parts, and thus can diffuse apart. This increases the distance between the fluorescent moieties, thus decreasing the amount of FRET that can occur between them. It is this decrease in FRET that is measured in the assay.

The assay described in Zlokarnik and U.S. Patent No. 5,741,657 can be modified
30 for use in the methods of the present invention by using an inducible promoter to drive β -lactamase where the promoter is activated by an intracellular signal generated by the opening or closing of a voltage-gated ion channel. Cells expressing a voltage-gated ion channel and the inducible promoter-driven β -lactamase are placed in the apparatus of the present invention, where the open or closed state of the voltage-gated ion channels can be controlled. The cells are
35 exposed to the cell-permeable dye and then exposed to substances suspected of being activators

or inhibitors of the voltage-gated ion channel. Those substances that cause a change in the open or closed state of the voltage-gated ion channel are identified by their effect on the inducible promoter-driven β -lactamase and thus on FRET. The inducible promoter-driven β -lactamase is engineered with a suitable promoter so that β -lactamase is induced when the substance is either
5 an activator or an inhibitor, depending upon the nature of the assay.

The flow of ions through voltage-gated ion channels can also be measured by measuring changes in membrane potential via the use of fluorescent voltage sensitive dyes. The changes in membrane potential will depend on the ion channels in the cell membrane. The resultant membrane potential will depend on the net properties of all the channels and the change
10 caused by inhibiting (through a substance that is an inhibitor or antagonist) or activating (through a substance that is an activator or an agonist) the voltage-gated ion channel of interest. One knowledgeable in cellular and membrane biophysics and electrophysiology will understand the directions of the changes in membrane potential since those changes depend on the ion channels present and the inhibition or activation of those channels by test substances. In many cases when
15 using fluorescent voltage sensitive dyes, the experimental system can be calibrated by using known activators or inhibitors of the voltage-gated ion channel of interest.

The present invention therefore includes assays that monitor changes in ion flow caused by activators or inhibitors of voltage-gated ion channels based upon FRET between a first and a second fluorescent dye where the first dye is bound to one side of the plasma membrane of
20 a cell expressing a voltage-gated ion channel of interest and the second dye is free to move from one face of the membrane to the other face in response to changes in membrane potential. In certain embodiments, the first dye is impenetrable to the plasma membrane of the cells and is bound predominately to the extracellular surface of the plasma membrane. The second dye is trapped within the plasma membrane but is free to diffuse within the membrane. At normal (*i.e.*,
25 negative) resting potentials of the membrane, the second dye is bound predominately to the inner surface of the extracellular face of the plasma membrane, thus placing the second dye in close proximity to the first dye. This close proximity allows for the generation of a large amount of FRET between the two dyes. Following membrane depolarization, the second dye moves from the extracellular face of the membrane to the intracellular face, thus increasing the distance
30 between the dyes. This increased distance results in a decrease in FRET, with a corresponding increase in fluorescent emission derived from the first dye and a corresponding decrease in the fluorescent emission from the second dye. See figure 1 of González & Tsien, 1997, Chemistry & Biology 4:269-277. See also González & Tsien, 1995, Biophys. J. 69:1272-1280 and U.S. Patent No. 5,661,035.

In certain embodiments, the first dye is a fluorescent lectin or a fluorescent phospholipid that acts as the fluorescent donor. Examples of such a first dye are: a coumarin-labeled phosphatidylethanolamine (*e.g.*, N-(6-chloro-7-hydroxy-2-oxo-2H--1-benzopyran-3-carboxamidoacetyl)-dimyristoylphosphatidyl-ethanolamine) or N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-dipalmitoylphosphatidylethanolamine); a fluorescently-labeled lectin (*e.g.*, fluorescein-labeled wheat germ agglutinin). In certain embodiments, the second dye is an oxonol that acts as the fluorescent acceptor. Examples of such a second dye are: bis(1,3-dialkyl-2-thiobarbiturate)trimethineoxonols (*e.g.*, bis(1,3-dihexyl-2-thiobarbiturate)trimethineoxonol) or pentamethineoxonol analogues (*e.g.*, bis(1,3-dihexyl-2-thiobarbiturate)pentamethineoxonol; or bis(1,3-dibutyl-2-thiobarbiturate)pentamethineoxonol). See González & Tsien, 1997, *Chemistry & Biology* 4:269-277 for methods of synthesizing various dyes suitable for use in the present invention. In certain embodiments, the assay may comprise a natural carotenoid, *e.g.*, astaxanthin, in order to reduce photodynamic damage due to singlet oxygen.

The use of such fluorescent dyes capable of moving from one face of the plasma membrane to the other is especially appropriate when the methods of the present invention are directed to inwardly rectifying potassium channels. Activation of inwardly rectifying potassium channels results in increased potassium current flow across the plasma membrane. This increased current flow results in a hyperpolarization of the cell membrane that can be detected by use of the technique described above since such hyperpolarization will result in greater FRET.

In particular embodiments of the present invention, cells are utilized that have been transfected with expression vectors comprising DNA that encodes a voltage-gated ion channel. Preferably, the cells do not naturally express corresponding voltage-gated ion channels. For example, if the expression vectors direct the expression of a voltage-gated calcium channel, the cells will not naturally express voltage-gated calcium channels. Alternatively, if the cells naturally express corresponding voltage-gated ion channels, those corresponding voltage-gated ion channels can be distinguished from the transfected voltage-gated ion channels in some manner, *e.g.*, by the use of appropriate inhibitors, by manipulation of membrane potential. A preferred cell line for use in the present invention is the HEK293 cell line (ATCC 1573) since this cell line naturally expresses endogenous potassium channels, which may be beneficial for electrical field stimulation experiments with channels that cause membrane potential depolarization (*e.g.*, sodium or calcium channels).

In a specific embodiment, the subject invention relates to a C1-6-37-3 cell and cell line. The C1-6-37-3 cell expresses the $\alpha 1C$ calcium ion channel subunit and the Kir 2.3 inward rectifying potassium channel on its plasma membrane.

Cells are generally eukaryotic cells, preferably mammalian cells. The cells may be grown to the appropriate number on the substrates or they may be placed on the substrate and used without further growth. The cells may be attached to the substrate or, in those embodiments where the cells are placed or grown in wells, the cells may be suspension cells that are suspended in the fluid in the wells. Primary cells or established cell lines may be used.

Suitable cells for transfection with expression vectors that direct the expression of voltage-gated ion channels include but are not limited to cell lines of human, bovine, porcine, monkey and rodent origin. The cells may be adherent or non-adherent. Cells and cell lines which are suitable and which are widely available, include but are not limited to: L cells L-M(TK⁻) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), HEK293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), CPAE (ATCC CCL 209), Saos-2 (ATCC HTB-85), ARPE-19 human retinal pigment epithelium (ATCC CRL-2302), GH3 cells, T-REx-293 cells (Invitrogen, R710-07), T-REx-CHO cells (Invitrogen, R718-07) and primary cardiac myocytes.

A variety of voltage-gated ion channels may be used in the present invention. For example, voltage-gated sodium channels, voltage-gated potassium channels, and voltage-gated calcium channels are suitable.

In certain embodiments of the present invention, the cells used do not naturally express the voltage-gated ion channel of interest. Instead, DNA encoding the voltage-gated ion channel is transfected into cells in order to express the voltage-gated ion channel in the plasma membrane of the cells. DNA encoding voltage-gated ion channels can be obtained by methods well known in the art. For example, a cDNA fragment encoding a voltage-gated ion channel can be isolated from a suitable cDNA library by using the polymerase chain reaction (PCR) employing suitable primer pairs. The cDNA fragment encoding the voltage-gated ion channel can then be cloned into a suitable expression vector. Primer pairs can be selected based upon the known DNA sequence of the voltage-gated ion channel it is desired to obtain. Suitable cDNA libraries can be made from cellular or tissue sources known to contain mRNA encoding the voltage-gated ion channel.

One skilled in the art would know that for certain voltage-gated ion channels, it is desirable to transfect, and thereby express, more than one subunit in order to obtain a functional voltage-gated ion channel. For example, N-type calcium channels are composed of a multisubunit complex containing at least an $\alpha 1B$, an $\alpha 2\delta$, and a $\beta 1$ subunit. On the other hand, T-type calcium channels are functional with only a single subunit, *e.g.*, $\alpha 1G$, $\alpha 1H$, or $\alpha 1I$.

Common knowledge in the art of the subunit composition of a voltage-gated ion channel of interest will lead the skilled artisan to express the correct subunits in the transfected cells. U.S. Patent No. 5,851,824 provides sequences for the α -1C/ α -1D, α -2, β -1, and γ .subunits

5 One skilled in the art could use published voltage-gated ion channel sequences to design PCR primers and published studies of voltage-gated ion channel expression to select the appropriate sources from which to make cDNA libraries in order to obtain DNA encoding the voltage-gated ion channels. The following publications may be of use in this regard:

U.S. Patent No. 5,876,958;

10 U.S. Patent No. 6,096,514;

U.S. Patent No. 6,090,623

Hondeghem, L.M., Katzung, B.G. (1984) Antiarrhythmic agents: the modulated receptor mechanism of action of sodium and calcium channel-blocking drugs. *Annu-Rev-Pharmacol-Toxicol.* 24:387-423.;

15 Zheng, W., Stoltefuss, J., Goldmann, S., and Triggle, D.J. (1992) Pharmacologic and radioligand binding studies of 1,4-dihydropyridines in rat cardiac and vascular preparations: stereoselectivity and voltage dependence of antagonist and activator interactions. *Mol. Pharmacol.* 41(3):535-541.; and

20 Triggle, D.J., Hawthorn, M.H. and Zheng, W. (1988) Potential-dependent interactions of nitrendipine and related 1,4-dihydropyridines in functional smooth muscle preparations. *J. Cardiovasc. Pharmacol.*, 12(Suppl.4):s91-s93.

The following table provides a list of known ion channels and information concerning each:

TABLE 1

Some ion channel genes of interest for ion flux experiments				
Symbol	Full Name	Cytogenetic Location	MIM Number	PubMed ID
SCN1	symbol withdrawn, see SCN1A			
SCN1A	sodium channel, voltage-gated, type I, alpha polypeptide	2q24	182389	8062593
SCN1B	sodium channel, voltage-gated, type I, beta polypeptide	19	600235	8394762
SCN2A1	sodium channel, voltage-gated, type II, alpha 1 polypeptide	2q22-q23	182390	1317301
SCN2A2	sodium channel, voltage-gated, type II, alpha 2 polypeptide	2q23-q24	601219	1317301
SCN2A	symbol withdrawn, see SCN2A1	-		
SCN2B	sodium channel, voltage-gated, type II, beta polypeptide	11q22-qter	601327	10198179
SCN3A	sodium channel, voltage-gated, type III, alpha polypeptide	2q24	182391	9589372
SCN4A	sodium channel, voltage-gated, type IV, alpha polypeptide	17q23-q25.3	603967	1654742
SCN4B	sodium channel, voltage-gated, type IV, beta polypeptide	reserved		
SCN5A	sodium channel, voltage-gated, type V, alpha polypeptide (long (electrocardiographic) QT syndrome 3)	3p21	600163	
SCN6A	sodium channel, voltage-gated, type VI, alpha polypeptide	2q21-q23	182392	10198179
SCN7A	symbol withdrawn, see SCN6A	-		
SCN8A	sodium channel, voltage gated, type VIII, alpha polypeptide	12q13.1	600702	7670495
SCN9A	sodium channel, voltage-gated, type IX, alpha polypeptide	2q24	603415	7720699
SCN10A	sodium channel, voltage-gated, type X, alpha polypeptide	3p21-p22	604427	9839820

TABLE 1 (Continued)

Some ion channel genes of interest for ion flux experiments				
Symbol	Full Name	Cytogenetic Location	MIM Number	PubMed ID
SCN11A	sodium channel, voltage-gated, type XI, alpha polypeptide	3p21-p24	604385	10444332
SCN12A	sodium channel, voltage-gated, type XII, alpha polypeptide	3p23-p21.3		10623608
SCNN1	symbol withdrawn, see SCNN1A	-		
SCNN1A	sodium channel, nonvoltage-gated 1 alpha	12p13	600228	7896277
SCNN1B	sodium channel, nonvoltage-gated 1, beta (Liddle syndrome)	16p12.2-p12.1		600760
SCNN1D	sodium channel, nonvoltage-gated 1, delta	1p36.3-p36.2	601328	8661065
SCNN1G	sodium channel, nonvoltage-gated 1, gamma	16p12	600761	7490094
CACNA1A	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	19p13	601011	8825650
CACNA1B	calcium channel, voltage-dependent, L type, alpha 1B subunit	9q34	601012	8825650
CACNA1C	calcium channel, voltage-dependent, L type, alpha 1C subunit	12pter-p13.2	114205	1650913
CACNA1D	calcium channel, voltage-dependent, L type, alpha 1D subunit	3p14.3	114206	1664412
CACNA1E	calcium channel, voltage-dependent, alpha 1E subunit	1q25-q31	601013	8388125
CACNA1F	calcium channel, voltage-dependent, alpha 1F subunit	Xp11.23-p11.22	300110	9344658
CACNA1G	calcium channel, voltage-dependent, alpha 1G subunit	17q22	604065	9495342
CACNA1H	calcium channel, voltage-dependent, alpha 1H subunit	16p13.3		9670923
CACNA1I	calcium channel, voltage-dependent, alpha 1I subunit	22q12.3-13.2		10454147

TABLE 1 (Continued)

Some ion channel genes of interest for ion flux experiments				
Symbol	Full Name	Cytogenetic Location	MIM Number	PubMed ID
CACNA1S	calcium channel, voltage-dependent, L type, alpha 1S subunit	1q31-q32	114208	7916735
CACNA2	symbol withdrawn, see CACNA2D1	-		
CACNA2D1	calcium channel, voltage-dependent, alpha 2/delta subunit 1	7q21-q22	114204	8188232
CACNA2D2	calcium channel, voltage-dependent, alpha 2/delta subunit 2	reserved		
CACNB1	calcium channel, voltage-dependent, beta 1 subunit	17q21-q22	114207	8381767
CACNB2	calcium channel, voltage-dependent, beta 2 subunit	10p12	600003	9254841
CACNB3	calcium channel, voltage-dependent, beta 3 subunit	12q13	601958	8119293
CACNB4	calcium channel, voltage-dependent, beta 4 subunit	2q22-q31	601949	9628818
CACNG1	calcium channel, voltage-dependent, gamma subunit 1	17q24	114209	8395940
CACNG2	calcium channel, voltage-dependent, gamma subunit 2	reserved	602911	
CACNG3	calcium channel, voltage-dependent, gamma subunit 3	reserved		
CACNG4	calcium channel, voltage-dependent, gamma subunit 4	17q24		10613843
CACNG5	calcium channel, voltage-dependent, gamma subunit 5	17q24		10613843
CACNG6	calcium channel, voltage-dependent, gamma subunit 6	19q13.4		11170751
CACNG7	calcium channel, voltage-dependent, gamma subunit 7	19q13.4		11170751
CACNG8	calcium channel, voltage-dependent, gamma subunit 8	19q13.4		11170751

TABLE 1 (Continued)

Some ion channel genes of interest for ion flux experiments				
Symbol	Full Name	Cytogenetic Location	MIM Number	PubMed ID
KCNA1	potassium voltage-gated channel, shaker-related subfamily, member 1 (episodic ataxia with myokymia)	12p13	176260	1349297
KCNA1B	literature alias, see KCNAB1	-		
KCNA2	potassium voltage-gated channel, shaker-related subfamily, member 2	12	176262	
KCNA2B	literature alias, see KCNAB2	-		
KCNA3	potassium voltage-gated channel, shaker-related subfamily, member 3	1p13.3 or 13	176263	2251283
KCNA3B	literature alias, see KCNAB3	-		
KCNA4	potassium voltage-gated channel, shaker-related subfamily, member 4	11p14	176266	2263489
KCNA4L	potassium voltage-gated channel, shaker-related subfamily, member 4-like	11q14		8449523
KCNA5	potassium voltage-gated channel, shaker-related subfamily, member 5	12	176267	
KCNA6	potassium voltage-gated channel, shaker-related subfamily, member 6	reserved	176257	
KCNA7	potassium voltage-gated channel, shaker-related subfamily, member 7	19	176268	
KCNA8	literature alias, see KCNQ1	-		
KCNA9	symbol withdrawn, see KCNQ1	-		
KCNA10	potassium voltage-gated channel, shaker-related subfamily, member 10	reserved	602420	
KCNAB1	potassium voltage-gated channel, shaker-related subfamily, beta member 1	3q26.1	601141	8838324
KCNAB2	potassium voltage-gated channel, shaker-related subfamily, beta member 2	1p36.3	601142	8838324
KCNAB3	potassium voltage-gated channel, shaker-related subfamily, beta member 3	17p13.1	604111	9857044

TABLE 1 (Continued)

Some ion channel genes of interest for ion flux experiments				
Symbol	Full Name	Cytogenetic Location	MIM Number	PubMed ID
KCNB1	potassium voltage-gated channel, Shab-related subfamily, member 1	20q13.2	600397	7774931
KCNB2	potassium voltage-gated channel, Shab-related subfamily, member 2	8		9612272
KCNC1	potassium voltage-gated channel, Shaw-related subfamily, member 1	11p15	176258	8449507
KCNC2	potassium voltage-gated channel, Shaw-related subfamily, member 2	12 and 19q13.4	176256	8111118
KCNC3	potassium voltage-gated channel, Shaw-related subfamily, member 3	19	176264	1740329
KCNC4	potassium voltage-gated channel, Shaw-related subfamily, member 4	1p21	176265	1920536
KCND1	potassium voltage-gated channel, Shal-related subfamily, member 1	Xp11.23-p11.3	300281	10729221
KCND2	potassium voltage-gated channel, Shal-related subfamily, member 2	7q31-32	605410	10551270
KCND3	potassium voltage-gated channel, Shal-related subfamily, member 3	1p13.2	605411	10942109
KCNE1	potassium voltage-gated channel, Isk-related family, member 1	21q22.1-q22.2	176261	8432548
KCNE1L	potassium voltage-gated channel, Isk-related family, member 1-like	Xq22.3	300328	10493825
KCNE2	potassium voltage-gated channel, Isk-related family, member 2	21q22.1	603796	10219239
KCNE3	potassium voltage-gated channel, Isk-related family, member 3	reserved	604433	10219239
KCNE4	potassium voltage-gated channel, Isk-related family, member 4	reserved		10219239
KCNF1	potassium voltage-gated channel, subfamily F, member 1	2p25	603787	9434767
KCNF2	literature alias, see KCNG2	-		

TABLE 1 (Continued)

Some ion channel genes of interest for ion flux experiments				
Symbol	Full Name	Cytogenetic Location	MIM Number	PubMed ID
KCNF	symbol withdrawn, see KCNF1	-		
KCNG1	potassium voltage-gated channel, subfamily G, member 1	20q13	603788	9434767
KCNG2	potassium voltage-gated channel, subfamily G, member 2	18q22-18q23	605696	10551266
KCNG	symbol withdrawn, see KCNG1	-		
KCNH1	potassium voltage-gated channel, subfamily H (eag-related), member 1	1q32-41	603305	9738473
KCNH2	potassium voltage-gated channel, subfamily H (eag-related), member 2	7q35-q36	152427	7842012
KCNH3	potassium voltage-gated channel, subfamily H (eag-related), member 3	12q13	604527	10455180
KCNH4	potassium voltage-gated channel, subfamily H (eag-related), member 4	reserved	604528	10455180
KCNH5	potassium voltage-gated channel, subfamily H (eag-related), member 5	14	605716	9738473
KCNIP1	Kv channel interacting protein 1	reserved		10676964
KCNIP2	Kv channel-interacting protein 2	10		10676964
KCNIP3	literature alias, see CSEN	-		
KCNJ1	potassium inwardly-rectifying channel, subfamily J, member 1	11q24	600359	7680431
KCNJ2	potassium inwardly-rectifying channel, subfamily J, member 2	17q23.1-q24.2	600681	7696590
KCNJ3	potassium inwardly-rectifying channel, subfamily J, member 3	2q24.1	601534	8088798
KCNJ4	potassium inwardly-rectifying channel, subfamily J, member 4	22q13.1	600504	8016146
KCNJ5	potassium inwardly-rectifying channel, subfamily J, member 5	11q24	600734	
KCNJ6	potassium inwardly-rectifying channel, subfamily J, member 6	21q22.1	600877	7796919

TABLE 1 (Continued)

Some ion channel genes of interest for ion flux experiments				
Symbol	Full Name	Cytogenetic Location	MIM Number	PubMed ID
KCNJ7	symbol withdrawn, see KCNJ6	-		
KCNJ8	potassium inwardly-rectifying channel, subfamily J, member 8	12p11.23	600935	8595887
KCNJ9	potassium inwardly-rectifying channel, subfamily J, member 9	1q21-1q23	600932	8575783
KCNJ10	potassium inwardly-rectifying channel, subfamily J, member 10	1q	602208	9367690
KCNJ11	potassium inwardly-rectifying channel, subfamily J, member 11	11p15.1	600937	7502040
KCNJ12	potassium inwardly-rectifying channel, subfamily J, member 12	17p11.1	602323	7859381
KCNJ13	potassium inwardly-rectifying channel, subfamily J, member 13	2q37	603208	9878260
KCNJ14	potassium inwardly-rectifying channel, subfamily J, member 14	19q13	603953	9592090
KCNJ15	potassium inwardly-rectifying channel, subfamily J, member 15	21q22.2	602106	9299242
KCNJ16	potassium inwardly-rectifying channel, subfamily J, member 16	17q23.1-q24.2	605722	11240146
KCNJN1	channel, subfamily J, inhibitor 1	17p11.2-p11.1	602604	8647284
KCNK1	potassium channel, subfamily K, member 1 (TWIK-1)	1q42-q43	601745	8661042
KCNK2	potassium channel, subfamily K, member 2 (TREK-1)	1q41	603219	9721223
KCNK3	potassium channel, subfamily K, member 3 (TASK-1)	2p23	603220	9312005
KCNK4	potassium inwardly-rectifying channel, subfamily K, member 4	11q13	605720	10767409
KCNK5	potassium channel, subfamily K, member 5 (TASK-2)	6p21	603493	9812978

TABLE 1 (Continued)

Some ion channel genes of interest for ion flux experiments				
Symbol	Full Name	Cytogenetic Location	MIM Number	PubMed ID
KCNK6	potassium channel, subfamily K, member 6 (TWIK-2)	19q13.1	603939	10075682
KCNK7	potassium channel, subfamily K, member 7	11q13	603940	10206991
KCNK9	potassium channel, subfamily K, member 9 (TASK-3)	8	605874	10734076
KCNK10	potassium channel, subfamily K, member 10	reserved	605873	
KCNK12	potassium channel, subfamily K, member 12	2p22-2p21		
KCNK13	potassium channel, subfamily K, member 13	14q24.1-14q24.3		11060316
KCNK14	potassium channel, subfamily K, member 14	2p22-2p21		11060316
KCNK15	potassium channel, subfamily K, member 15	reserved		
KCNMA1	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	10	600150	7987297
KCNMB1	potassium large conductance calcium-activated channel, subfamily M, beta member 1	5q34	603951	8799178
KCNMB2	symbol withdrawn, see KCNMB3	-		
KCNMB2	potassium large conductance calcium-activated channel, subfamily M, beta member 2	reserved	605214	10097176
KCNMB2L	symbol withdrawn, see KCNMB3L	-		
KCNMB3	potassium large conductance calcium-activated channel, subfamily M beta member 3	3q26.3-q27	605222	10585773

TABLE 1 (Continued)

Some ion channel genes of interest for ion flux experiments				
Symbol	Full Name	Cytogenetic Location	MIM Number	PubMed ID
KCNMB3L	potassium large conductance calcium-activated channel, subfamily M, beta member 3-like	22q11		10585773
KCNMB4	potassium large conductance calcium-activated channel, subfamily M, beta member 4	reserved	605223	
KCNMBL	symbol withdrawn, see KCNMB3	-		
KCNMBLP	symbol withdrawn, see KCNMB3L	-		
KCNN1	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 1	19p13.1	602982	8781233
KCNN2	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	reserved	605879	
KCNN3	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3	22q11-q13.1	602983	9491810
KCNN4	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	19q13.2	602754	9380751
KCNQ1	potassium voltage-gated channel, KQT-like subfamily, member 1	11p15.5	192500	8528244
KCNQ1OT1	KCNQ1 overlapping transcript 1	11p15.5	604115	10220444
KCNQ2	potassium voltage-gated channel, KQT-like subfamily, member 2	20q13.3-2 20q13.3	121200	9425895
KCNQ3	potassium voltage-gated channel, KQT-like subfamily, member 3	8q24	121201	9425900
KCNQ4	potassium voltage-gated channel, KQT-like subfamily, member 4	1p34	603537	10025409
KCNQ5	potassium voltage-gated channel, KQT-like subfamily, member 5	6q14		10787416

TABLE 1 (Continued)

Some ion channel genes of interest for ion flux experiments				
Symbol	Full Name	Cytogenetic Location	MIM Number	PubMed ID
KCNS1	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 1	reserved	602905	9305895
KCNS2	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 2	8q22	602906	9305895
KCNS3	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 3	reserved	603888	10484328

PCR reactions can be carried out with a variety of thermostable enzymes including but not limited to AmpliTaq, AmpliTaq Gold, or Vent polymerase. For AmpliTaq, reactions can be carried out in 10 mM Tris-Cl, pH 8.3, 2.0 mM MgCl₂, 200 μ M of each dNTP, 50 mM KCl, 0.2 μ M of each primer, 10 ng of DNA template, 0.05 units/ μ l of AmpliTaq. The reactions are heated at 95°C for 3 minutes and then cycled 35 times using suitable cycling parameters, including, but not limited to, 95°C, 20 seconds, 62°C, 20 seconds, 72°C, 3 minutes. In addition to these conditions, a variety of suitable PCR protocols can be found in PCR Primer, A Laboratory Manual, edited by C.W. Dieffenbach and G.S. Dveksler, 1995, Cold Spring Harbor Laboratory Press; or PCR Protocols: A Guide to Methods and Applications, Michael et al., eds., 1990, Academic Press.

It is desirable to sequence the DNA encoding voltage-gated ion channels obtained by the herein-described methods, in order to verify that the desired voltage-gated ion channel has in fact been obtained and that no unexpected changes have been introduced into its sequence by the PCR reactions. The DNA can be cloned into suitable cloning vectors or expression vectors, e.g., the mammalian expression vector pcDNA3.1 (Invitrogen, San Diego, CA) or other expression vectors known in the art or described herein.

A variety of expression vectors can be used to recombinantly express DNA encoding voltage-gated ion channels for use in the present invention. Commercially available expression vectors which are suitable include, but are not limited to, pMC1neo (Stratagene), pSG5 (Stratagene), pcDNAI and pcDNAIamp, pcDNA3, pcDNA3.1, pCR3.1 (Invitrogen, San Diego, CA), EBO-pSV2-neo (ATCC 37593), pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pCI.neo (Promega), pTRE (Clontech, Palo Alto, CA), pV1Neo, pIRESneo (Clontech, Palo Alto, CA), pCEP4 (Invitrogen, San Diego, CA), pSC11, and pSV2-dhfr (ATCC 37146). The choice of

vector will depend upon cell type in which it is desired to express the voltage-gated ion channels, as well as on the level of expression desired, and the like.

The expression vectors can be used to transiently express or stably express the voltage-gated ion channels. The transient expression or stable expression of transfected DNA is well known in the art. See, *e.g.*, Ausubel et al., 1995, "Introduction of DNA into mammalian cells," in Current Protocols in Molecular Biology, sections 9.5.1-9.5.6 (John Wiley & Sons, Inc.).

As an alternative to the above-described PCR methods, cDNA clones encoding ion channels can be isolated from cDNA libraries using as a probe oligonucleotides specific for the desired voltage-gated ion channels and methods well known in the art for screening cDNA libraries with oligonucleotide probes. Such methods are described in, *e.g.*, Sambrook *et al.*, 1989, *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, *DNA Cloning: A Practical Approach*, MRL Press, Ltd., Oxford, U.K., Vol. I, II. Oligonucleotides that are specific for particular voltage-gated ion channels and that can be used to screen cDNA libraries can be readily designed based upon the known DNA sequences of the voltage-gated ion channels and can be synthesized by methods well-known in the art.

EXAMPLE 1

Immunofluorescence staining was all performed at room temperature. Cells were washed three times with Dulbecco's phosphate buffered saline (D-PBS) and then fixed with 4% paraformaldehyde for 30 min. After three washes with D-PBS, the cells were blocked and permeabilized with TBS (10 mM Tris-HCl, pH 7.5, and 150 mM NaCl) containing 4% nonfat dry milk and 0.1 % Triton X-100 for 1 hr, and incubated with the affinity purified polyclonal antibodies against human alpha 1C or kir2.3 for 1 hr. Then the cells were washed three times with TBS and incubated with the secondary antibody (Cy3-conjugated anti-rabbit IgG, at 1:250, Jackson ImmunoResearch, PA) for 1 hr. The cells were finally washed with D-PBS three times and viewed under indirect immunofluorescence on a Zeiss Axioskop microscope. Figures 1 and 2 show that cells were successfully transfected and expressing calcium and potassium channels, respectively, on their plasma membranes.

EXAMPLE 2

Hek 293 cells were stably transfected with the alpha 1C subunit of the L-type Calcium ion channel and Kir 2.3 inward K⁺ rectifying channel (C1-6-37-3 cells). Calcium influx into the cells was measured in a FLIPR™ (Molecular Devices, CA). The C1-6-37-3 cells were seeded into black 96 well plates with clear bottoms coated with poly-D-lysine at density of 50000 cells/well and cultured overnight. Next day the cells were washed twice with assay buffer containing 137 mM NaCl; 0.34 mM Na₂HPO₄; 4.2 mM NaHCO₃; 0.44 mM KH₂PO₄; 0.41 mM MgSO₄; 0.49 mM MgCl₂; 20 mM HEPES; 5.5 mM D-glucose and 0.1% BSA and incubated with Fluo-3AM (final concentration 4 μM, Molecular probe) for 1 hr at 37°C, 5% CO₂ and 95% O₂. After cells were washed four times either with resting condition (5.8 K⁺) or depolarized condition (30 mM K⁺), the cell plate was placed into the FLIPR™ to monitor cell fluorescence (λ_{EX}=488 nm, λ_{EM}=540 nm) before and after the addition of calcium blockers and agonists (final 85.8 mM K⁺).

Cellular membrane potentials were measured using an Axopatch 200B patch amplifier (Axon Instruments Inc., Foster City, CA) in current clamp mode using the "perforated patch" clamp method (Horn and Korn). The patch pipette contained (in mM): 120 KMeSO₄, 20 KCl, 9 Mg₂Cl, 10 HEPES, Nystatin 200 μg/ml, pH 7.3. The bath solution contained (in mM): 140 NaCl, 1.2 Mg₂Cl, 10 HEPES, 1.3 Ca₂Cl, 21 D-glucose, pH 7.4. Standard electrophysiological methods were employed. Changes in extracellular potassium were made by additions of a concentrated stock to the standard bath solution to the appropriate dilution.

Results:

Table 2 shows the membrane potential of the C1-6-37-3 cells recorded at various extracellular potassium concentrations. This experiment confirms that changes in potassium alter the membrane potential of these cells approximately as predicted by the Nernst equation.

Figure 4 shows that calcium influx into fluo-3 loaded cells in response to increasing potassium concentration was concentration dependent and possessed an EC₅₀ of 11 mM K⁺. The potency of the inhibitory effect of nimodipine and other calcium channel antagonists on calcium influx through the α₁C channel was shown to depend on membrane potential (table 3, Figures 5-7). Preincubation of cells with 30 mM K⁺ (V_m = -28 mV) increased the potency of nimodipine to block calcium influx compared to the preincubation of these cells with 5.8 mM K⁺ (V_m = -65 mV). This assay captures the state-dependent interactions of 1,4-dihydropyridines that have been identified previously.

Table 2. Membrane potential of C1-6-37-3 cell line recorded in various potassium concentrations using Nystatin perforated patch

[K] _{out} mM	Resting potential	n
0.4	-99.3 ± 10.6	6
4.0	-73 ± 0.7	6
5.8	-64.7 ± 2.6	7
30	-27.6 ± 2.4	7
80	7.5 ± 7.1	7

5 Values are the mean ±

Table 3. IC₅₀ (nM) values of calcium channel antagonists for inhibition of K⁺-induced calcium influx either in 30 mM K⁺ (depolarized condition) or 5.8 mM K⁺ (resting condition).

Antagonists	5.8 mM [K] _o	n	30 mM [K] _o	n	F
Nimodipine	59 ± 27	4	3 ± 3	5	21
Nifedipine	43 ± 12	4	7 ± 1	3	7
Nitrendipine	51 ± 18	4	6 ± 3	2	8
Mibefradil	3458 ± 867	4	791 ± 43	5	4

10 Values are the mean ± S.D.

F indicates the ratio of the IC₅₀ values of 5.8 mM K⁺ and 30 mM K⁺.

EXAMPLE 3

Cellular membrane potentials were measured using an Axopatch 200B patch amplifier (Axon Instruments Inc., Foster City, CA) in current clamp mode using the 'perforated patch' clamp method (Horn and Korn). The patch pipette contained (in mM): 120 KMeSO₄, 20 KCl, 9 Mg₂Cl, 10 HEPES, Nystatin 200 µg/ml, pH7.3. The bath solution contained (in mM): 140 NaCl, 1.2 Mg₂Cl, 10 HEPES, 1.3 Ca₂Cl, 21 D-glucose, pH7.4. Standard electrophysiological methods were employed. Changes in extracellular potassium were made by additions of a concentrated stock to the standard bath solution to the appropriate dilution. Figure 3 shows the relationship between extracellular potassium ([K]_o) and cell membrane potential. Three situations are shown. One is the prediction of the Nernst equation for a perfectly K-selective membrane. The other curves show the effects of partial permeability by other ions,

Na⁺ and/or Cl⁻. Membrane potential can be set in a non-voltage clamped cell by adjusting external potassium. A cell line expressing an inward rectifier K channel (Kir2.3) to set the resting membrane potential will permit control of membrane resting potential by extracellular potassium.

5 Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties to the extent not inconsistent with the teachings herein. All patents, patent applications, publications, texts and references discussed or cited herein are understood to be incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually set forth in its entirety. In addition, all references, 10 patents, applications, and other documents cited in an Invention Disclosure Statement, Examiner's Summary of Cited References, or otherwise entered into the file history of this application are taken to be incorporated by reference into this specification for the benefit of later applications claiming priority to this application. Finally, all terms not specifically defined are first taken to have the meaning given through usage in this disclosure, and if no such meaning is 15 inferable, their normal meaning.